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Detection of point mutations in DNA using capillary electrophoresis in a polymer network

Aldy W. H. M. Kuypers

Department of Internal Medicine, Division of Hematology, University Hospital Nijmegen, Geert Grooteplein Zuid 8, 6525 GA Nijmegen (Netherlands), and Laboratory of Instrumental Analysis, Department of Chemistry, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven (Netherlands)

Peter M. W. Willems

Department of Internal Medicine, Division of Hematology, University Hospital Nijmegen, Geert Grooteplein Zuid 8, 6525 GA Nijmegen (Netherlands)

Marcel J. van der Schans

Laboratory of Instrumental Analysis, Department of Chemistry, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven (Netherlands)

Peter C. M. Linssen and Hans M. C. Wessels

Department of Internal Medicine, Division of Hematology, University Hospital Nijmegen, Geert Grooteplein Zuid 8, 6525 GA Nijmegen (Netherlands)

Chris H. M. M. de Bruijn and Frans M. Everaerts

Laboratory of Instrumental Analysis, Department of Chemistry, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven (Netherlands)

Ewald J. B. M. Mensink*

Department of Internal Medicine, Division of Hematology, University Hospital Nijmegen, Geert Grooteplein Zuid 8, 6525 GA Nijmegen (Netherlands)

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ABSTRACT

The use of capillary electrophoresis (CE) in a polymer network for single-strand conformation polymorphism (SSCP) is investigated. SSCP is a method to detect DNA point mutations, essential in the diagnosis of several diseases. The PCR (polymerase chain reaction) amplified p53 gene, a tumour suppressor gene known to be frequently mutated in malignant cells, was subjected to CE analysis. Two single-strand DNA fragments of 372 bp in length differing in only one nucleotide could be separated. We conclude that SSCP using CE in a polymer network is a powerful method for the detection of point mutations in DNA sequences.

* Corresponding author.

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INTRODUCTION

Point mutations are nucleotide changes in both complementary strands of the deoxyribonucleic acid (DNA) molecule, the carrier of genetic information. When a mutation occurs in the germline DNA present in a gamete and interferes with the function of genes, it can result in a genetic transmissible disease. During recent years, a lot of information was generated about DNA mutations involved in the multistep nature of cancer. Cancer is a distinct type of genetic disease in which not one, but several mutations are required. Each mutation causes a wave of cellular multiplication resulting in gradual increases in tumour size, disorganisation and malignancy. Three to six such mutations appear to be required to complete this process [1]. Part of these mutations are point mutations in the DNA, others are chromosomal aberrations like translocations, deletions, amplifications and inversions. The detection of DNA point mutations might be helpful for the diagnosis of genetic diseases and cancer.

Point mutations can be detected by several techniques, most of them use slab gel analysis. To increase the sensitivity of these techniques, DNA fragments can be multiplied prior to analysis by enzymatic amplification, using the polymerase chain reaction (PCR) [reviewed in ref. 2]. Because mutations alter the resistance of the DNA segments to denaturation resulting in changes in mobility on gradient gels [3], the denaturing gradient gel electrophoresis (DGGE) technique allows double stranded amplified DNA products that differ by one or more mutations to be separated on acrylamide gels cast with linear gradients of denaturants. Alternatively, localisation of point mutations in PCR amplified products is also possible by a method using chemical modification. In this method heteroduplexes are generated between normal and mutated single strand DNA molecules. A nucleotide mismatch will be produced at the site of the mutation. Mismatched cytosine or thymine residues are sensitive to modification by hydroxylamine or osmium tetroxide ("HOT") respectively. After this modification, the DNA heteroduplex is sensitive to piperidine cleavage. Cleavage products of different lengths can be separated on a denaturing acrylamide gel [4]. A third and more simple method for the detection of nucleotide substitutions is the single-strand conformation polymorphism (SSCP) technique. Point mutations give rise to conformational changes of the single stranded DNAs and lead to a mobility shift of the DNA fragments on neutral polyacrylamide gels. This SSCP technique is a convenient and efficient method for the detection of point mutations [5].

The techniques mentioned above merely detect small changes in the DNA but they do not provide information about the nature of these changes. For this, DNA sequencing is the appropriate technique [6].

The fast development of applications using capillary electrophoresis, a family of related techniques that use narrow capillaries to perform high efficiency separations of molecules [reviewed in ref. 7], were a reason for us to investigate whether SSCP of DNA molecules could also be detected using capillary electrophoresis (CE) in a polymer network instead of the traditional polyacrylamide gel electrophoresis (PAGE).

We studied whether we could detect point mutations in the p53 gene located on the short arm of chromosome 17 [8]. This gene is known to be frequently mutated in malignancies and can therefore be used as a molecular marker for (pre-) malignant cells [9]. The p53 gene contains five evolutionary conserved regions and the mutations are clustered in region II–V [10]. As a model, we used DNA derived from the cell line CEM and from patients with multiple myeloma [11].

We present the use of CE for the detection of small DNA changes. This CE application is compared with the use of conventional slab gel electrophoresis. As will be demonstrated, the advantages of the CE method are numerous compared to other techniques used for the detection of point mutations. CE opens the possibility for regular screening of patient samples, which is important for the diagnosis of several diseases.

EXPERIMENTAL

DNA isolation

DNA was isolated [12] from normal white blood cells derived from a pool of 30 normal persons, from the heterozygous cell line CEM which contains a p53 point mutation in one of its alleles the other being normal, and from bone marrow cells of two multiple myeloma patients known to have a p53 point mutation. The patients and the cell line material were the same as those used in the study by Willems *et al.* [11].

Amplification of the p53 mutation clusters A and B by polymerase chain reaction (PCR)

Primers used for amplification of the mutation clusters A and B of the p53 gene were synthesised on a 391A DNA synthesiser (Applied Biosystems, Warrington, UK). The names and the sequences of the primers used were: p53-i3-primer; 5′-TTT CTT TGC TGC CGT GTT CCA and p53-AS1-primer; 3′-ATA AGA TGC TGA GGA GGG GC. Positions of the 5′ end of the primers in the p53 sequence are 12973 and 13344 respectively [13]. The 100-μl PCR mixture contained 30 pmoles of each primer, 250 μM dNTP's and 2.5 U *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD, USA). PCR was performed for 35 cycles at 95°C for 1 min, at 56°C for 1 min and at 72°C for 1 min in a Perkin-Elmer Cetus thermocycler (Perkin-Elmer, Norwalk, CT, USA). The length of the generated PCR fragment was checked on a 2% agarose gel.

Preparation of PCR samples prior to CE analysis

The PCR products were precipitated with ethanol, rinsed with 70% ethanol to lower the salt concentration, and dissolved in TBE (90 mM Tris-borate pH 8.3, 0.2 mM EDTA). Prior to SSCP analysis the samples were heated to 90°C for 3 min, snap-cooled and kept on ice-water for 10 min, to melt the double stranded DNA molecules into single stranded before electrokinetic injection onto the column filled with a polymer network.

Capillary electrophoresis

A fused silica capillary (310 mm × 0.75 mm I.D.) was precoated with 3-methacryloylpropylmethoxysilane. The coated capillary was mounted in an assembly cartridge and placed in a Bio-Rad BioFocus 3000 CE instrument (Bio-Rad, Hercules, CA, USA). Additional coating was performed by purging for 5 min with a 4% acrylamide without crosslinker (4% T, 0% C) solution. The acrylamide was left in the capillary for 1 h to polymerise using 8 μl 10% N,N,N′,N′-tetramethylethylenediamine (TEMED) and 4 μl 10% ammonium persulfate (APS) per ml acrylamide solution [14]. The thus prepared capillary could be used for at least 20 runs using the following protocol. After each run the capillary was rinsed with water for 2 min and filled again with the polymerised 4% linear polyacrylamide network by purging for 5 min using 8 bar pressure. The polyacrylamide network contained TAE buffer (40 mM Tris-acetate pH 8.3, 2 mM EDTA). Before electrokinetic injection of the sample the inlet end of the capillary was dipped in water to prevent contamination of the sample. Injections were performed at reversed polarity (cathode at the injection side) of 167 V/cm for 10 or 20 s and separations were performed at reversed polarity under constant voltage of 250 V/cm, in TAE running buffer (same concentration as above). Buffers were degassed by bubbling helium through the solutions for 15 min. SSCP analyses were performed using UV absorption at 260 nm for detection. During the run the temperature of the caroussel was kept at 15°C and the capillary was kept at 25°C.

The molecular mass marker used was pBr322 DNA digested by the Hae III restriction enzyme (DNA MW marker V, Boehringer Mannheim, Germany), resulting in fragment sizes of 8, 11, 18, 21, 51, 57, 64, 80, 89, 104, 123, 124, 184, 192, 213, 234, 267, 434, 458, 504, 540 and 587 bps.

Fractionation

With the BioFocus 3000 Integrator program (Bio-Rad) the peaks on the electropherogram of a test run were integrated. In the fractionation run samples were collected in a vial containing 10 μl TAE running buffer every 15 s during 3 min starting 1 min before the first peak. To avoid con-

tamination, a 0.01-min sampling wash step was simulated in a new vial containing 500 μl TAE running buffer after each fraction. An aliquot of 5 μl of all fractions was directly used as template in a 25 cycles PCR using the same conditions as described before. The PCR products were not ethanol precipitated before CE re-analysis.

RESULTS

Analysis of single-strand conformation polymorphisms of amplified DNA fragments by capillary electrophoresis in a polymer network

DNA amplification of the point mutation clusters A and B of the p53 gene resulted in a DNA fragment of 372 base pairs. This corresponds to the expected size based upon the p53 sequence [13]. The PCR product was tested on a conventional 2% agarose gel (data not shown) and on a capillary filled with a linear 4% polyacrylamide network. We used the migration times of a pBr322 Hae III DNA restriction fragment marker (Fig. 1a) to compare the migration time of the double stranded PCR fragment (Fig. 1b) with. Peaks in the electropherograms of PCR fragments at migration times of *ca.* 8 to 12 min are due to DNA primers, salt, enzyme and nucleotides used in the PCR.

Fig. 1c shows the electropherogram of the single stranded PCR fragments of normal white blood cells after denaturation of the DNA. Because of the complementary nucleotide sequence of the two single strands of one DNA molecule, their folded conformation will be different. This obviously leads to different mobilities in the polyacrylamide network and results in two different

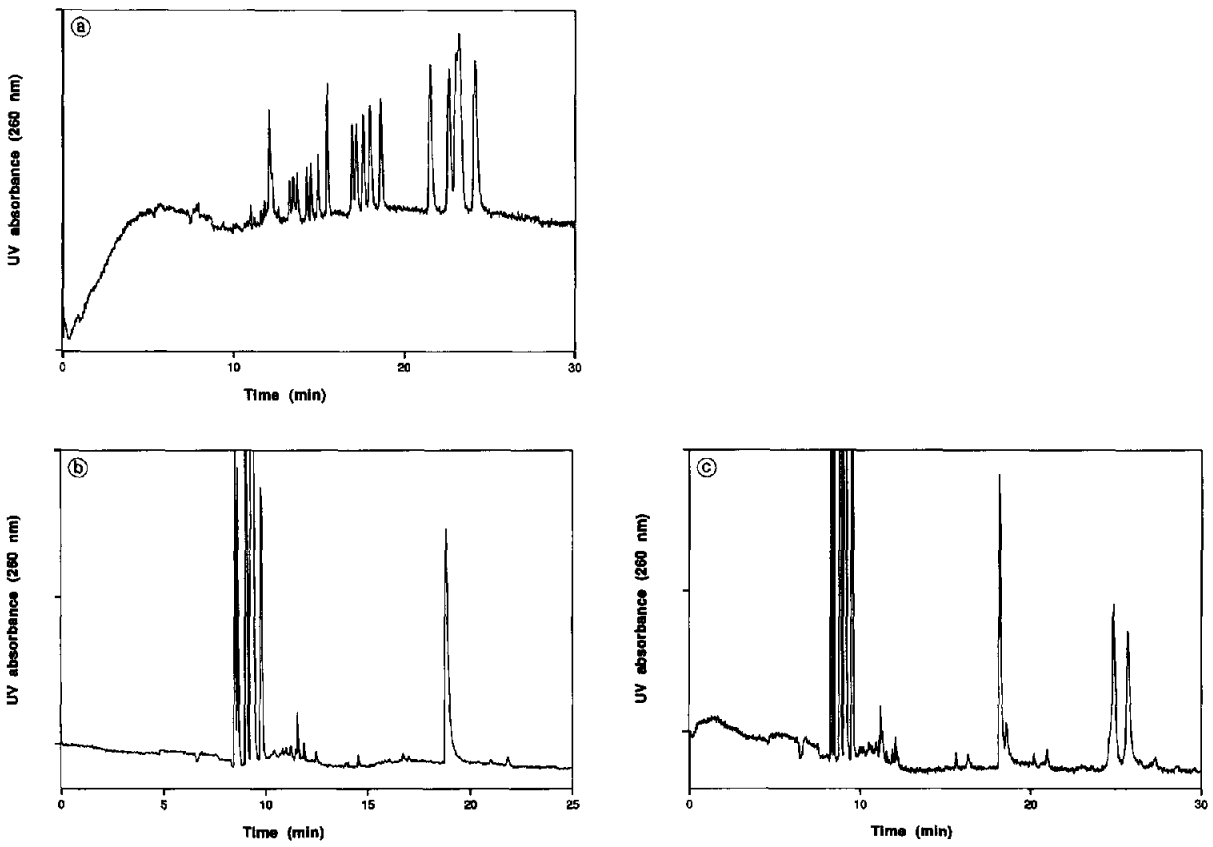

Fig. 1. (a) Electropherogram of a pBr322 Hae III DNA molecular mass marker with fragment lengths: 8, 11, 18, 21, 51, 57, 64, 80, 89, 104, 123, 124, 184, 192, 213, 234, 267, 434, 458, 504, 540, 587 bp. (b) Electropherogram of a double stranded p53 AB amplified fragment (372 bp) of normal DNA. (c) Electropherogram of a denatured p53 AB amplified fragment of normal DNA.

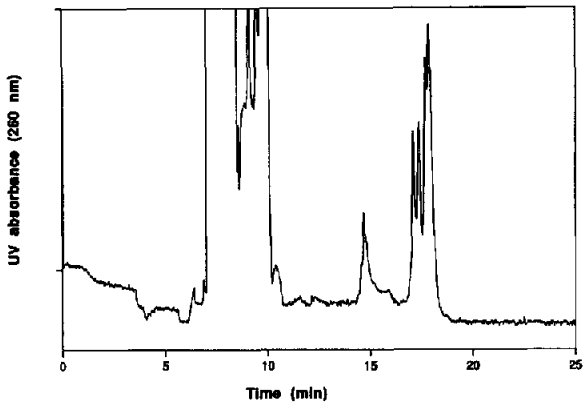

Fig. 2. Electropherogram of the p53 AB amplified fragment of cell line CEM, after denaturation. Migration time of the double strand peak is 14.70 min. Migration time of the single strand peak 1 is 17.11, of peak 2 is 17.39, of peak 3 is 17.70 and of peak 4 is 17.87 min.

peaks at 24.86 and 25.71 min on the electropherogram. The double stranded DNA peak at 18.18 min still present in the denatured sample is due to incomplete denaturation of the double stranded DNA and renaturation of both single strand fragments in the vial before loading.

With the conditions for the analysis of the heterozygous cell line CEM used in this study, apart from the double stranded DNA peak at 14.70 min, four other fragments are visible on the electropherogram at 17.11, 17.39, 17.70 and 17.87 min, indicating the presence of more than one DNA sequence (Fig. 2).

Fig. 3 shows the separation of the PCR fragments of multiple myeloma patients 1 and 2 known to have a point mutation in the AB mutation cluster of the p53 gene [11]. The double stranded DNA fragments derived from the patients have the same size as the fragment derived from normal DNA which was checked on agarose gel (data not shown). After denaturation, the electropherogram shows more than two single stranded peaks, again indicating the presence of more than one DNA sequence.

Analysis of fractionated peaks by capillary electrophoresis in a polymer network

To find out whether the different peaks observed in the electropherogram of cell line CEM corresponded to different sequences, all the fifteen collected fractions were re-amplified by PCR. All the amplified fractions showed an amplification product on agarose gel, whereas no amplified DNA was present in the negative control reaction in which water was used instead of DNA template (data not shown). Results of CE analysis of re-amplified fractions 5 and 8, corresponding to peak 1 (17.11 min in Fig. 2) and peak 2 (17.39 min in Fig. 2), are shown in Fig. 4a and b. In a PCR amplification of the fractionated single strand peak, also its complementary sequence

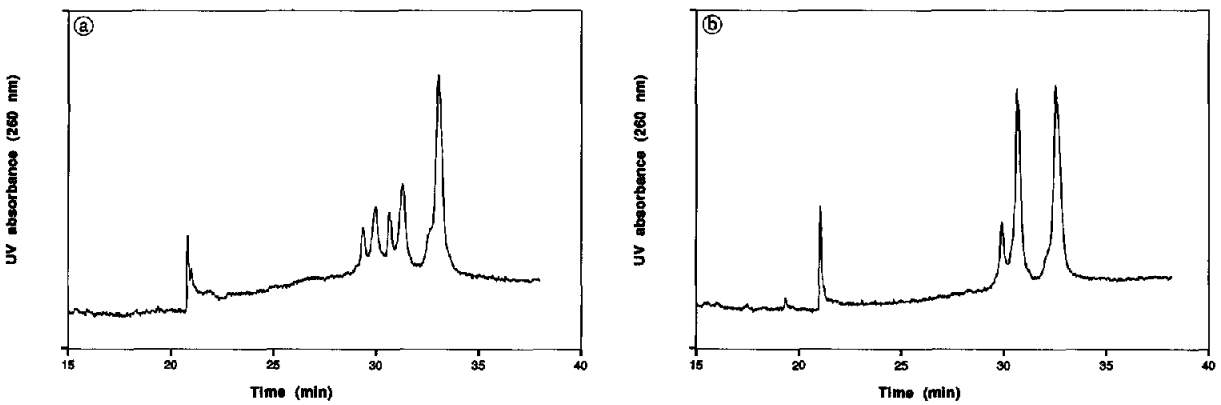

Fig. 3. Electropherogram of the p53 AB amplified fragment of patients 1 (a) and 2 (b) after denaturation.

is generated and amplified. This leads to two single strand peaks on the electropherogram. CE analysis of a mixture of fractions 5 and 8 is shown in Fig. 4c. The fractions 5 and 8 give rise

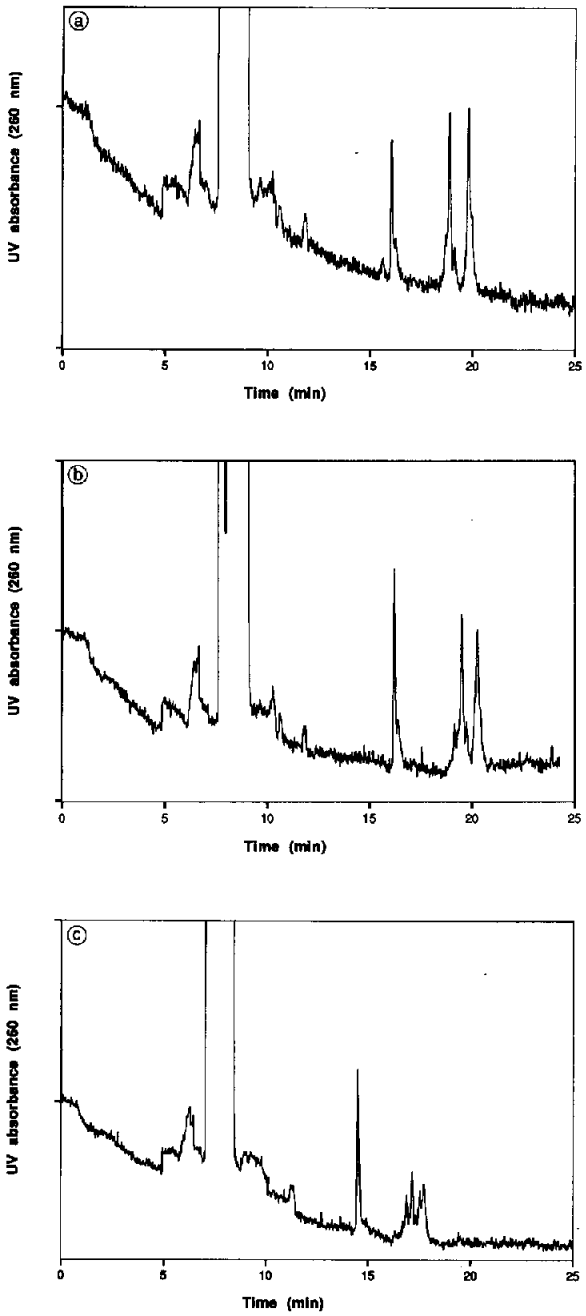

Fig. 4. The electropherograms of single strand peak 1 and peak 2 (see Fig. 2) after PCR amplification and denaturation are shown in (a) and (b), respectively. The electropherogram of a mixture of peak 1 and 2 is shown in (c).

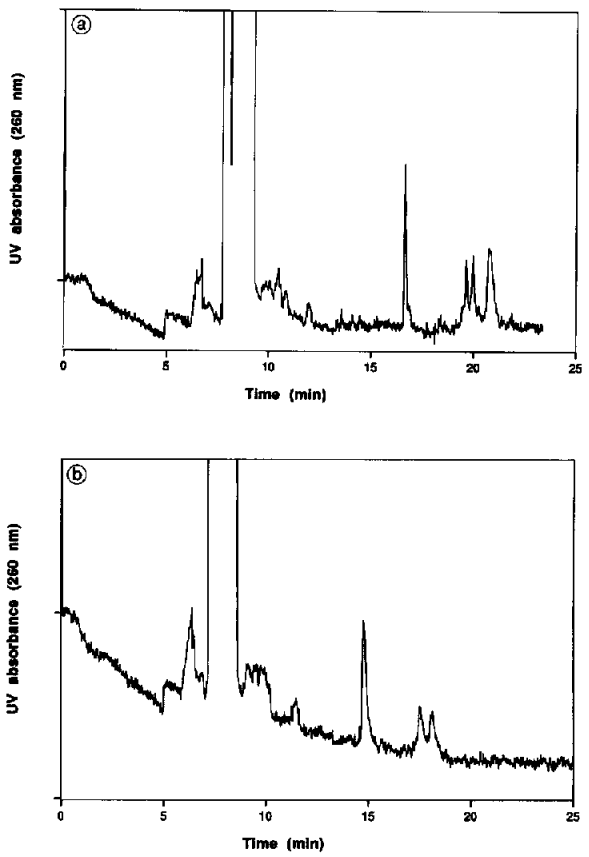

Fig. 5. Electropherogram of a mixture of amplified normal DNA and amplified peak 1 (a) and of amplified normal DNA and amplified peak 2 (b) after denaturation.

to different electrophoretic patterns and therefore four peaks are observed. This indicates that the different peaks indeed contained different p53 sequences. CE analysis of a mixture of fraction 5 and normal DNA shows three single strand peaks and a mixture of normal DNA and fraction 8 shows only two single strand peaks (Fig. 5a and 5b).

DISCUSSION

We decided to investigate the feasibility of detection of single strand conformation polymorphism (SSCP) of DNA molecules using capillary electrophoresis (CE) in a polymer network because of several advantages of SSCP over the chemical modification or the denaturing gradient

gel electrophoresis method. First, SSCP analysis is performed on a simple non-denaturing gel which is easy to make. Second, the technique can be used in a preparative way. Mutated DNA strands can be re-amplified and subjected to further analysis. Third, for the detection of point mutations the presence of normal DNA in the same sample is not necessary as in the chemical modification method.

We tested the technique on the cell line CEM (Fig. 2) and patients 1 and 2 (Fig. 3). If a test sample showed a different number of peaks on a SSCP–CE electropherogram compared with the normal control sample, we concluded that the sample contained a (point)mutation. The cell line CEM contains one normal and one point mutated allele of the p53 gene as shown before [11]. We used CE in a preparative way to prove that the different peaks represent different DNA sequences. DNA was obtained by fractionation followed by PCR amplification and a secondary CE analysis. This way DNA obtained from sampling of peak 1 was shown to have its sequential counterpart in peak 3. The same holds for peak 2 and 4. By comparison of these electrophoretic patterns with normal DNA we conclude that the DNA represented by peaks 1 and 3 contained the p53 point mutation (Fig. 5a).

The patients 1 and 2 are known to have a point mutation in the AB mutation cluster of the p53 gene [11]. Because the p53 mutation exists only in the malignant cells in a background of normal cells the electropherogram should reveal more than the two single strand peaks of the normal cells. Fig. 3 indeed shows more than two peaks indicating the presence of point mutated malignant cells.

Our results show that, using CE in a polymer network, it is possible to separate two single stranded DNA molecules of the same length differing in just one nucleotide. Compared to the detection of point mutations by the SSCP technique using conventional slab gels, SSCP–CE has great advantages. It is less laborious, the time used per sample for analysis is much shorter (30 min *vs.* several h) and the amount of sample needed for analysis is very small (far more than 100 CE analyses can be done with one PCR). An important additional advantage of SSCP–CE is that the detection is non-radioactive and the commercially available systems are automated. Other systems for non-radioactive SSCP analyses exist, but these systems are not automated, only able to run a few samples at a time, and the staining of gels takes additional time [15,16]. UV detection of SSCP patterns by CE is direct and data are stored as an electropherogram which can be used for re-evaluation, for example for quantification. Another important advantage of SSCP–CE analysis is the possibility to use the technique in a preparative way in combination with PCR, as shown above. Ultimate prove for the presence of point mutations is delivered by re-analysis of different fractions or by DNA sequencing.

A problem in this study was the fact that the PCR amplified fractions collected before and after the single strand peaks and used as negative controls also were positive on agarose gel. Secondary CE analysis showed four peaks (data not shown). Because the PCR using water instead of DNA template was shown to be negative, contamination in the PCR could be excluded. We conclude that in all the samples collected by fractionation a background of DNA was present, even when the sample was collected from the base line. This background did not interfere with our analysis since the DNA collected from a UV peak on the electropherogram was strongly enriched for one sequence and that sequence was mainly amplified in the PCR (Fig. 4). Whether vigorous rinsing and cleaning steps can prevent this contamination remains to be studied.

It is known that the conformation of a DNA fragment in non-denaturing polyacrylamide gels depends strongly on the percentage of acrylamide, the temperature and the presence or absence of glycerol. Therefore, for screening of DNA from which it is not known whether it contains point mutations it is necessary to perform the SSCP analysis under more than one condition to minimise false negative results [5]. With CE using linear polyacrylamide networks it is easy to purge, wash and re-fill the capillary and run different conditions subsequently and in an

automated way. In this study we only used PCR fragments known to have a p53 mutation so it was not necessary to use more than one condition to detect all mutations. Under the temperature and polymer network conditions used no false positive or negative SSCP results were observed in repeated runs.

We detected point mutations in fragments of 372 bp. Using conventional slab-gel SSCP point mutations could be detected in fragments differing in size from *ca.* 100 to 400 bp [17,18]. Whether point mutations in fragments beyond these size limits can be detected using CE needs to be studied. How the sensitivity (*i.e.* the minimum amount of mutated DNA that can be detected in relation to the amount of normal DNA) of the CE relates to conventional slab-gel SSCP analysis is as yet unknown. Moreover, whether the resolution of the SSCP–CE can be increased in such a way that every DNA mutation can be detected in a single run remains a topic for further study as well.

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